sequence divergence is slightly higher among the different geographic groups than within the same group, which may reflect random drift or adaptation to their respective local environments (Figure 1). *Thermus flavus*, *Thermus filiformis* Tok4A2, *Thermus filiformis* Tok6A1, *Thermus sp.* SM32, *Thermus sp.* Vil3, *Thermus aquaticus* YT-1, and *Thermus sp.* AK16D (SEQ. ID. No. 14) ligases shared 98.2%, 89.9%, 89.5%, 89.8%, 88.3%, 88.2%, 88.1% with *Thermus thermophilus* HB8 DNA ligase, respectively. The adenylation site of the enzymes (^{118}KXDG where X is in general a hydrophobic residue), as identified by site-directed mutagenesis of *Tth* DNA ligase, is completely identical among all *Thermus* ligases, furthermore, the flanking sequences of the adenylation motif are also identical except *Tsp.* AK16D in which the aa residues ^{117}H before the ^{118}K is substituted by an ^{118}R (Figure 1B). In non-*Thermus* NAD^+-dependent ligases discovered to date, the corresponding position is either a Pro or a Leu. The two isolates from Japan can be distinguished from the other *Thermus* strains by a 3-aa-insertion at position 234.

Example 8 - Cloning, Expression And Purification Of DNA Ligase From *Tsp.* AK16D

To maximize the chance of finding a *Thermus* ligase with novel properties, *Tsp.* AK16D ligase was chosen which showed the least sequence identity as compared with *T. thermophilus* ligase. To obtain the complete sequence of the ORF (i.e. open reading frame), the fragments of the N- and C-terminus of the gene were amplified by inverse PCR and were subject to direct sequencing. The complete ORF of the *Thermus sp.* AK16D ligase gene consists of 674 amino acids, as compared to 676 aa for *Tth* ligase and 674 aa for T. scot ligase (Figure 1C). The full-length *Thermus sp.* AK16D ligase gene was PCR amplified using *Pfu* polymerase and cloned into expression plasmid pET11c (Novagen). The integrity of the insert containing the ligase gene was verified by DNA sequencing. The pET11c plasmid expressing *Tsp.* AK16D ligase was transformed into competent *E. coli* cells NovaBlue(DE3)pLysS. Production of ligases was induced by adding IPTG to 1 mM final concentration. *Tsp.* AK16D ligase protein was expressed to approximately 10% of total cellular proteins (Figure 2, lane 3). Heating at 70°C for 15 minutes denatured most of *E. coli* proteins while leaving the thermostable ligases as the dominant band (Figure 2, lane 4). A

cibacron blue based affinity chromatography (Pharmacia) further removed residual *E. coli* proteins and nucleic acids, yielding apparently homogenous *Tsp.* AK16D ligase protein as judged by Coomassie staining (Figure 2, lane 5).

Example 9 - Salt, pH, and NAD^+ Dependence Of The Ligation Reaction

Figure 3A depicts the pH dependence of ligase activity of *Tth* and *Tsp.* AK16D ligase proteins. The shape of the pH dependence curves of *Tth* ligase and *Tsp.* AK16D ligase is essentially superimposable. The optimal pH is 8.5 for both *Tth* ligase and *Tsp.* AK16D ligase with greater than 80% activity observed between pH 7.8 and 9.5. The identity of pH effect suggests that both of the ligases possess similar local environment at their catalytic center, which is in agreement with the degree of sequence conservation between the two ligases. Figure 3B depicts the salt concentration dependence of ligase activity of *Tth* and *Tsp.* AK16D ligase proteins. The optimum KCl concentration for *Tth* ligase and *Tsp.* AK16D ligase are 100 and 50 mM, respectively. Figure 3C depicts the NAD^+ concentration dependence of ligase activity of *Tth* and *Tsp.* AK16D proteins. The optimum NAD^+ concentration is 1 mM for both *Tth* ligase and *Tsp.* AK16D ligase. The similarity of the NAD profiles is in keeping with the highly conserved nature of the N-terminal domain of the ligases which is involved in NAD^+ binding.

Example 10 - Effects Of Divalent Metals On The Ligation Reaction

Divalent metal ion is indispensable for each of the three steps in a ligation reaction: (i) adenylation of a lysine residue in the adenylation motif KXDG; (ii) transfer of the adenylate to the 5' phosphate to form a DNA-adenylate intermediate; and (iii) formation of the phosphodiester bond with the release of adenosine monophosphate (AMP). In general, Mg^{2+} is the preferred metal ion for both ATP-dependent and NAD^+-dependent ligases. Mg^{2+} was substituted with alkaline earth metal ion Ca^{2+} and commonly studied period 4 transition metal ions. *Tth* and *Tsp.* AK16D ligases could use Mn^{2+} as an alternative metal cofactor to support ligation activity (Figure 4). Both enzymes were less active with Ca^{2+}, while Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} failed to support ligation. In comparison, ATP-dependent

ligase from Hin (i.e. *Haemophilus influenzae*) uses only Mg^{2+} and Mn^{2+} as the metal cofactor for nick closure but not Ca^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} (Cheng, et al., Nucleic Acids Res, 25(7):1369-1374 (1997), which is hereby incorporated by reference); ATP-dependent ligase from *Chlorella* virus PBCV-1 can use Mg^{2+}, Mn^{2+}, and Co^{2+} but not Ca^{2+}, Cu^{2+}, and Zn^{2+} (Ho, et al., J Virol, 71(3):1931-1937 (1997), which is hereby incorporated by reference). Using Ca^{2+} as the metal cofactor, *Thermus* enzymes were able to convert most of the substrate into the DNA-adenylate intermediate. However, the rates of nick closure were reduced which led to the accumulation of the DNA-adenylate intermediate (Figure 4B). A small amount of the intermediate was observed with Ni^{2+}; however, ligation product was not observed at the current detection level, suggesting that Ni^{2+} could not support the nick closure step (Figure 4B). To further compare the relative activity of the two *Thermus* ligases with Mg^{2+} and Mn^{2+}, the generation of ligation product was first monitored over a 20-min time period. As shown in Figure 5, the *Thermus* enzymes were consistently more active with Mg^{2+} than with Mn^{2+}. Second, ligation activity up to 40 mM Mg^{2+} or Mn^{2+} concentrations (Figure 6) was assayed. Both of the enzymes responded sensitively to the change of the metal ion concentration in the reaction mixture. At high M^{2+} concentrations, the high ionic strength may inhibit the enzyme activity, consistent with KCl dependence profile (Figure 4). Similar to the time-course results, the *Thermus* enzymes were more active with Mg^{2+} than with Mn^{2+} (Figure 6). The discrepancy on the relative activity of *Thermus* ligases between this study and an earlier report may be due to use here of cloned enzymes while the earlier work used purified native enzyme (Takahashi, et al., J Biol Chem, 259(16):10041-10047 (1984), which is hereby incorporated by reference).

Example 11 - Steady State Kinetics

The steady state kinetic constants were measured by monitoring the formation of fluorescently labeled ligation product over time using substrate concentrations spanning estimated *K*m values (Table 1).